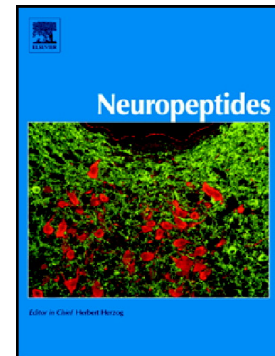


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Effects of RVD-hemopressin (α) on feeding and body weight after standard or cafeteria diet in rats

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Abstract

Palatability and variety of foods are major reasons for hedonic eating, and hence for obesity. Hemopressin, a hemoglobin α chain-derived peptide, plays antagonist/inverse agonist role on cannabinoid (CB)₁ receptors, while RVD-hemopressin(α)[RVD-hp(α)], a N-terminally extended form of hemopressin, has been reported as an allosteric modulator of CB₁ and CB₂ receptors.

We investigated the effects of 14 daily intraperitoneal injections of RVD-hp(α), in *Sprague-Dawley* rats fed a highly palatable cafeteria-style (CAF) diet (30% fat, 56% carbohydrate, 14% protein; 4.20 kcal/g) compared to standard laboratory chow (STD) food (3.5% fat, 63% carbohydrate, 14% protein, 19.5% other components without caloric value; 3.20 kcal).

Food intake, body weight and locomotor activity were recorded throughout the study. Finally, rats were sacrificed and agouti-related peptide (AgRP), neuropeptide Y (NPY), pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) gene expression in the hypothalamus was measured by real-time reverse transcription polymerase chain reaction.

We found that CAF diet increased food intake as compared to STD diet. In both STD and CAF diet fed rats, RVD-hp(α) treatment inhibited food intake, increased locomotor activity but did not modify body weight. In vehicle injected animals, CAF as compared to STD diet increased AgRP mRNA gene expression. RVD-hp(α) treatment decreased POMC mRNA levels in both diet groups and lowered the elevated AgRP levels induced by CAF diet.

RVD-hp(α) treatment plays an anorexigenic role paralleled by increased locomotor activity both in STD and CAF diet fed rats. The inhibition of feeding could be partially mediated by lowering of hypothalamic POMC and AgRP gene expression levels.

Keywords:

RVD-hemopressin(α)

Food intake

Cafeteria diet

Abbreviations:

Agouti-related peptide (AgRP); Cafeteria diet (CAF); Cocaine- and amphetamine-regulated transcript (CART); Cannabinoid 1 (CB1); Endocannabinoid (eCB); fatty acid amide hydrolase (FAAH); Hemopressin (Hp); Neuropeptide Y (NPY); Pro-opiomelanocortin (POMC); RVD-hemopressin(α) [RVD-hp(α)]; standard laboratory chow (STD); transient receptor potential vanilloid 1 (TRPV1).

1. Introduction

The hypothalamus plays a pivotal role in the control of food intake and energy balance (Rui, 2013), while the hedonic aspects related to food intake are processed by corticolimbic reward pathways, particularly in the amygdala (Balleine et al., 2003). Overeating is stimulated by the abundance of food, particularly highly palatable, energy-rich food, such as sugar and chocolate (Balleine et al., 2003; Berthoud, 2004; De Castro and Stroebele, 2002; Rolls, 2003; Tordoff, 2002; Ulijasz, 2002). Palatability is reputed the most important non-homeostatic factor controlling food intake (Berthoud 2004). Enhanced taste and flavor can elicit further food intake in satiated animals, and chronically lead to obesity (Sclafani, 1987; Warwick and Schiffman, 1992). Studies also point to palatable meal in inducing food addiction (Erlanson-Albertsson, 2005; Colantuoni et al., 2002; Gosnell and Krahn 1992; Nestle and Aghajanian, 1997). Actually, palatable food intake stimulates neuronal pathways that are also affected by abuse substances, such as alcohol and morphine (Volkow and Wise, 2005; Berridge, 1996; Gosnell, 2000; Kelley et al., 2003). Endogenous cannabinoids and cannabinoid agonists have a well established role of appetite stimulators, by affecting mesolimbic and hypothalamic circuitries involved in feeding-related reward (Ramos et al., 2005, Balleine et al., 2003). In particular, CB1 antagonists and inverse agonists inhibit reward for sucrose pellets or chocolate-flavored food, blocking compulsive eating of palatable food in rats (Warwick and Schiffman, 1992; Droste et al., 2010; Dore et al., 2014).

Hemopressin, a nonapeptide derived from the α_1 -chain of rat hemoglobin, and RVD-hemopressin-(α) [RVD-hp(α)], a N-terminally extended form of hemopressin, bind to CB1 receptors, playing the role of antagonist/inverse agonist and negative allosteric

modulator, respectively (Heimann et al., 2007; Bauer et al., 2012; Han et al., 2014). On the other hand, both VD-hemopressin- α [VD-hp(α)], an other N-terminally extended hemopressin, and RVD-hp(α) were firstly reported as CB1 agonists *in vitro* (Gomes et al., 2009). In addition, *in vivo*, VD-hp(α) and RVD-hp(α) functioned as CB1 agonists (Han et al., 2014; Tanaka et al., 2014). Recent findings revealed that RVD-hp(α) behaved as a positive allosteric modulator of CB2 receptor (Petrucchi et al., 2017). Han and collaborators (2014) reported that central injection of VD-hp(α) increased food intake and decreased locomotor activity in mice, consistently with its putative agonist CB1 activity. However, RVD-hp(α) has been reported to exert anorexigenic effects after both repeated peripheral and single central administration, in rats, which could be related, albeit partially, to its negative allosteric modulator activity on CB1 receptors (Ferrante et al, 2017; Recinella et al., 2018). In addition, we previously reported that RVD-hp(α) did not modify locomotor activity, after a single peripheral administration in rats (Leone et al., 2017). Anxiolytic/antidepressant effects have also been induced by RVD-hp(α), after both central and peripheral administration in rats (Dodd et al., 2010; Ferrante et al., 2017; Leone et al., 2017; Recinella et al., 2018). In the present work, we have further investigated the anorexigenic effects induced by RVD-hp(α) administration in rats fed a highly palatable cafeteria-style diet (CAF), compared to standard laboratory chow diet (STD).

2. Materials and methods

2.1 Animals

Male adult *Sprague-Dawley* rats (180-200 g, 7-8 weeks of age) were housed in Plexiglas cages (2 animals per cage; 40 cm × 25 cm × 15 cm) with a millboard changed every 12 h and maintained under standard laboratory conditions (22 ± 1 °C; 60% humidity), on a 12 h/12 h light/dark cycle (light phase: 07:00–19:00 h), with free access to tap water and food. Housing conditions and experimentation procedures were strictly in accordance with the European Community ethical regulations (EU Directive 2010/63/EU) on the care of animals for scientific research. All the procedures were approved by local Ethical Committee of G. d'Annunzio University and by Italian Ministry of Health (Ministry authorization n. 880/24th August 2015).

2.2 Experimental procedure

After 1-week acclimatization, rats (n=48) were randomized to standard (STD) diet (n=24) or cafeteria (CAF) diet (n=24) for 14 days. STD diet rats were fed laboratory chow in pellets (3.5% fat, 63% carbohydrate, 14% protein, 19.5% other components without caloric value; 3.20 kcal/g). CAF diet rats were given, in addition to the standard chow as above, cafeteria-style food (into two separate troughs), which included chips of parmigiano-reggiano cheese, potato chips, roasted, hazelnuts, cookies, curls of salt butter and bits of torrone chocolate, in the ratio 1:1:1:1:1:1 (in terms of grams). The average composition of CAF diet was: 30% fat, 56% carbohydrate, and 14% protein, 4.20 kcal/g (Tab. 1) (Brunetti et al., 2009; Ottani et al., 2007). Both STD and CAF diet fed rats were divided into two groups of 12 animals each and injected daily for 14 days intraperitoneally, as previously described (Brunetti et al., 2009; Ottani et al., 2007), at 9.00 am, during the light phase, with either vehicle (0.2 ml saline) or RVD-hp(α) (0.2

ml, 10 nmol/rat). RVD-hp(α) was diluted in saline and dosage was selected on the basis of previous studies (Dodd et al., 2010, 2013; Ferrante et al., 2017; Leone et al., 2017). Food intake (expressed in kilocalories) and body weight were recorded every 4 days throughout the study in all study groups. Food eaten through the whole period was recorded in each group of rats, by subtracting residual food in the dispenser and hick millboard from the food initially given. The cafeteria-style food has been weighed as single component. Soon after each injection, rectal temperature measurements were taken once every hour, over a five-hour period (10:00–15:00), by the insertion of a plastic-coated thermocouple into the rectum (pd 0331; Panlab, Barcelona, Spain) (0, 8, 20, 28 days).

24 h after the last RVD-hp(α) administration, the animals were sacrificed by CO₂ inhalation (100 % CO₂ at a flow rate of 20 % of the chamber volume per minute).

Fat depots were quickly excised and weighed, after animal sacrifice. Body fat content represents the total wet weight of retroperitoneal and epididymal fat normalized by body weight (g/100 g body weight).

RVD-hp(α) has been synthesized and characterized in our laboratories, as previously reported (Mollica et al., 2013, 2015a and 2015b).

2.3 Locomotor activity

Immediately after each injection, locomotor activity in the home cage was recorded by a video camera positioned in the top-centre of the cage and connected to a computer, as previously reported (Leone et al., 2015). Separate video frames with a highly accurate, programmable, monochrome frame grabber board (DataTranslationTM, type DT3153). The apparatuses were purchased from 2 Biological Instruments (Besozzo VA, Italy). Intelligent software Smart version 2.5 (Panlab, sl Bioresearch and Technology,

Barcelona, Spain) was used for data processing, automatically recording distance travelled (cm), number of Z-beam breaks, for 10 min.

2.4 RNA extraction, reverse transcription and real-time reverse transcription polymerase chain reaction (real-time RT PCR)

Immediately after sacrifice, brains were collected and hypothalami rapidly removed [using the landmarks of the optic chiasm rostrally and the mammillary bodies caudally (Xu et al., 2011); coordinates of hypothalamus as follows: anteroposterior (AP), 2.06 mm; mediolateral (ML), 0.2 mm; dorsoventral (DV), - 6.89 mm (Paxinos and Watson, 2007)], dissected and stored in RNAlater solution (Ambion, Austin, TX) at -20 °C until further processed. Total RNA was extracted from the hypothalamus using TRI Reagent (Sigma-Aldrich, St. Louis, MO), as previously reported (Brunetti et al., 2014). One µg of total RNA extracted from each sample in a 20 µl reaction volume was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Reactions were incubated in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) initially at 25°C for 10 min, then at 37°C for 120 min, and finally at 85°C for 5 s. Gene expression was determined by quantitative real-time PCR using TaqMan probe-based chemistry (Assays-on-Demand Gene Expression Products, Rn00567382_m1 for CART gene, Rn00595020_m1 for POMC gene, Rn01431703_g1 for AgRP gene, Rn00561681_m1 for NPY gene, Rn00577086_m1 for FAAH gene). β -actin (Applied Biosystems, Foster City, CA, USA, Part No. 4352340E) was used as the housekeeping gene. The real-time PCR was carried out in triplicate for each cDNA sample in relation to each of the investigated genes. Data were elaborated with the Sequence Detection System (SDS) software version 2.3 (Applied Biosystems,

Foster City, CA, USA). Gene expression was relatively quantified by the comparative $2^{-\Delta\Delta C_t}$ method (Livak et al., 2001).

2.5 *In vitro studies: TRPV1 channel assay.*

HEK293 (human embryonic kidney) cells stably over-expressing recombinant human TRPV1 were grown on 100 mm diameter Petri dishes as mono-layers in minimum essential medium (EMEM) supplemented with non-essential amino acids, 10% foetal bovine serum, and 2 mM glutamine, and maintained at 5% CO₂ at 37 °C. The effect of the substances on intracellular Ca²⁺ concentration ([Ca²⁺]_i) was determined by using Fluo-4, a selective intracellular fluorescent probe for Ca²⁺ accordingly to Schiano and De Petrocellis (2016). Briefly, on the day of the experiment, cells were loaded at room temperature for 1 h with the methyl ester Fluo-4-AM (Invitrogen) in EMEM, then washed twice in Tyrode's buffer (145 mM NaCl, 2.5 mM KCl, 1.5 mM CaCl₂, 1.2 mM MgCl₂, 10mM D-glucose, and 10 mM HEPES, pH 7.4), resuspended in the same buffer, and transferred (about 100,000 cells) to the quartz cuvette of the spectrofluorimeter (Perkin-Elmer LS50B PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) under continuous stirring. The changes in [Ca²⁺]_i were determined before and after the addition of various concentrations of test compounds by measuring cell fluorescence (λ_{ex} = 488 nm, λ_{em} = 516 nm) at 25 °C. Curve fitting (sigmoidal dose–response variable slope) and parameter estimation were performed with GraphPad Prism® (GraphPad Software Inc., San Diego, CA). Potency was expressed as the concentration of test substances exerting a half-maximal agonist effect (i.e. half-maximal increases in [Ca²⁺]_i) (EC₅₀). The efficacy of the agonists was first determined by normalizing their effect to the maximum Ca²⁺ influx effect on [Ca²⁺]_i observed with application of 4 μ M ionomycin (Invitrogen).

2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Food intake (kcal), body weight (g) and locomotor activity data were collected from each of the animals used in the experimental procedure and means \pm S.E.M. were determined for each experimental group and analyzed by two way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test. Gene expression data were collected from each of the animals used in the experimental procedure and means \pm S.E.M. were determined for each experimental group and analyzed by one way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test. As for gene expression analysis, 1.00 (calibrator sample) was considered the theoretical mean for the comparison. Statistical significance was accepted at $p < 0.05$, $p < 0.005$ and $p < 0.001$. As regards to the animals randomized for each experimental group, the number was calculated on the basis of the “Resource Equation” $N=(E+T)/T$ ($10 \leq E \leq 20$), where E, N and T represent the number of degrees of freedom in an ANOVA, animals and treatments, respectively (Charan and Kantharia, 2013).

3. Results

Figures 1 and 2 (see also Tables 2 and 3 reporting means \pm S.E.M.) show food intake recorded every 4 days and mean daily food intake expressed in kilocalories (kcal), respectively, in rats fed with either STD or CAF diet, and treated with vehicle or RVD-hp(α). The caloric intake in animals fed with CAF diet is significantly higher [$F_{4/13} = 3.18$; $p = 0.001$] with respect to STD rats. RVD-hp(α) (10 nmol) treatment induced inhibition of food intake in both STD [$F_{11/5} = 3.33$; $p = 0.003$] and CAF [$F_{6/8} = 3.58$; $p = 0.04$] animals, since day 12 and day 20, respectively. As for CAF diet, we observed that RVD-hp(α) treatment did not modify the chow/mixture ratio as compared to CAF-vehicle (Figure 3).

On the other hand, we found that the effect was weaker in CAF rats [$F_{5/16} = 2.85$; $p = 0.003$].

Figures 4 and 5 (see also Tables 4 and 5 reporting means \pm S.E.M.) show body weight recorded every 4 days and body weight gain expressed in grams, respectively, in rats fed with either STD or CAF diet, and treated with vehicle or RVD-hp(α). Independently of treatment, CAF rats had increased body weight gain [$F_{7/11} = 3.01$; $p = 0.000$] as compared to STD rats, while RVD-hp(α) treatment did not modify body weight in both CAF and STD groups. Moreover, we evaluated fat mass (g/100 g body weight) in rats fed with either STD or CAF diet, and treated with vehicle or RVD-hp(α), finding a significant increase in CAF animals compared to STD [fat mass (g/100 g body weight): means \pm S.E.M., STD-vehicle 10.0 ± 0.7 ; CAF-vehicle 13.76 ± 0.1 , $F_{8/19} = 2.48$; $p = 0.005$]. On the other hand, we have not found any difference between STD-RVD-hp(α) and CAF- RVD-hp(α).

Additionally, no difference in rectal body temperature was also found in rats fed with either STD or CAF diet, and treated with vehicle or RVD-hp(α). [body temperature: means \pm S.E.M., Day 0: STD-vehicle 37.65 ± 0.20 ; CAF-vehicle 37.43 ± 0.22 ; STD-RVD-hp(α) 38.20 ± 0.23 ; CAF- RVD-hp(α) 37.80 ± 0.22 ; Day 8: STD-vehicle 37.65 ± 0.20 ; CAF-vehicle 37.43 ± 0.22 ; STD-RVD-hp(α) 38.20 ± 0.23 ; CAF- RVD-hp(α) 37.80 ± 0.22 ; Day 20: STD-vehicle 38.50 ± 0.34 ; CAF-vehicle 37.90 ± 0.13 ; STD-RVD-hp(α) 37.75 ± 0.37 ; CAF- RVD-hp(α) 38.23 ± 0.39 ; Day 28: STD-vehicle 38.20 ± 0.17 ; CAF-vehicle 38.10 ± 0.03 ; STD-RVD-hp(α) 37.90 ± 0.11 ; CAF- RVD-hp(α) 38.03 ± 0.11].

In vehicle injected animals, despite increased caloric intake in CAF animals, we did not observed any effect of CAF vs. STD diet on locomotor activity. Moreover, we found that RVD-hp(α) treatment induced a significant increase of horizontal and vertical activity [$F_{3/18} = 3.16$; $p = 0.002$; $F_{5/16} = 2.85$; $p = 0.003$] both in STD and CAF diet rats (Fig. 6) (see also Table 6 reporting means \pm S.E.M.).

In the hypothalamus of vehicle treated animals, CAF diet resulted in increased AgRP mRNA levels and no significant differences in CART, POMC and NPY gene expression levels, as compared to STD diet. In STD rats, RVD-hp(α) treatment inhibited POMC gene expression, with no effects on CART, AgRP and NPY mRNA levels. In CAF rats, RVD-hp(α) treatment also inhibited POMC gene expression. Interestingly, the elevated AgRP mRNA levels found in CAF rats injected with vehicle were lowered by RVD-hp(α) treatment [$F_{2/23} = 3.42$; $p = 0.001$] (Fig. 7; see also Table 7 reporting means \pm S.E.M.). Also, in CAF rats CART and NPY mRNA levels were not affected by RVD-hp(α) (Fig. 5).

Finally, we observed that RVD-hemopressin(α) was able to induce intracellular Ca^{2+} elevation in HEK293 cells stably transfected with the human TRPV1 cDNAs. The calcium influx was expressed as percentage response (21 ± 0.5 %), compared to the Ca^{2+} ionophore ionomycin 4 μM . The related EC_{50} value was 32.6 ± 2.5 μM . Five minute preincubation of human TRPV1-HEK293 cells with the strong TRPV1 antagonist 5-iodoresiniferatoxin (0.1 μM), had no effect at all on RVD-hemopressin(α) response (Fig. 8). On the other hand, we cannot exclude that Ca^{2+} influx, although not TRPV1-dependent, could however induce the synthesis on demand of anandamide. To explore this hypothesis we evaluated the effect of the peptide on anandamide metabolism, *in vivo*. We found that peptide injection decreased FAAH gene expression in the hypothalamus (Fig. 9).

4. Discussion

Increased availability of highly palatable food is considered a major contributor to eating disorders and obesity. A highly palatable diet induces a spontaneous and progressively increasing hyperphagia and body weight gain in animals (Judge et al., 2008), which could be modulated by endogenous cannabinoids (Lau et al., 2017).

As expected, we found that in CAF diet fed rats the caloric intake was significantly higher compared to STD diet, paralleled by an increase in body weight gain. Our present findings show that CAF diet is attractive for rats and mimicks conditions leading to overfeeding in humans.

RVD-hp(α) treatment inhibited food intake both in STD and CAF diet rats, but the effect was weaker in latter. We can speculate that the more palatable CAF diet can drive macronutrient selection toward more energy dense food, blunting the anorectic effect of RVD-hp(α). Similar effects were reported for other anorexigenic substances such as obestatin, tested in CAF vs. STD diet fed animals (Brunetti et al., 2009). Although inducing decreased caloric intake, RVD-hp(α) treatment did not modify body weight, which could probably relate to decreased uncoupling protein 1 (UCP-1) gene expression in brown adipose tissue and consequent reduction of thermogenic energy expenditures in RVD-hp(α) treated animals (Ferrante et al., 2017).

Also, we did not observed any effect of CAF vs. STD diet on locomotor activity, as reported by da Costa Estrela and collaborators (2015). However, we found increased locomotor activity induced by RVD-hp(α) treatment both in STD and CAF animals.

Increased locomotion was reported for cannabidiol, which is also a CB1 receptor negative allosteric modulator, and stimulates locomotor activity in animal models (Britch et al., 2017).

Interestingly, the increased energy expenditure induced by locomotion could counter the inhibitory effect on uncoupling protein 1 (UCP-1) gene expression in brown adipose tissue following RVD-hp(α) administration (Ferrante et al., 2017), explaining the observed null effect of RVD-hp(α) on body weight. The lack of any effect on body temperature is in agreement with the downregulation of UCP-1 gene expression following peptide administration (Ferrante et al., 2017).

If, on one side, we can't exclude that the acute increase in locomotor activity is followed by a significant decrease in locomotion in later time points, on the other hand, the only way to determine the underlying cause of the lack of weight change is to measure energy expenditure. The lack on body weight change following RVD-hp(α) administration could indicate a minor role displayed by the peptide on obesity therapy. Nevertheless, the antidepressant and anti-anxiety effects found in our previous studies (Leone et al., 2017; Recinella et al., 2018) are an intriguing result that could account for further deepening.

The observed effects induced by CAF diet and RVD-hp(α) treatment could be mediated by modifications of neuropeptide levels in the hypothalamus. In vehicle treated animals, the finding of increased feeding induced by CAF diet could be related to elevated AgRP mRNA levels in the hypothalamus, as compared to vehicle injected animals. AgRP plays a well established role in stimulating feeding. Previous studies reported that AgRP injection increased the intake of a palatable diet, possibly through the modulation of pre-ingestive factors, such as taste and palatability, or post-ingestive factors such as gut peptide release (Tracy et al., 2007). Also, Lazzarino and collaborators (2017) observed, in rats fed a CAF diet, increased AgRP gene expression in the ventromedial and paraventricular hypothalamus with a concomitant reduction of AgRP gene expression in

arcuate nucleus. On the other hand, we observed that treatment of CAF rats with RVD-hp(α) resulted in lowering of the elevated AgRP mRNA to levels found in STD animals, partially explaining the anorexigenic effects induced by the peptide.

A further mechanistic effect underlying decreased feeding induced by RVD-hp(α) is the observed inhibition of hypothalamic POMC gene expression in both STD and CAF animals. Recent studies suggest that the orexigenic effects of endocannabinoids could be mediated by increased POMC-derived β -endorphin (Bakkali-Kassem et al., 2011). The negative allosteric modulation of CB1 receptors induced by RVD-hp(α) could induce anorexigenic effects, both in STD and CAF rats, through reduced β -endorphin levels consequent to inhibited POMC gene expression. In this context, the reduction of POMC gene expression, despite appearing paradoxical, is in agreement with the interaction of RVD-hp(α) with CB1 receptor. Previous studies indicated that feeding stimulating effects induced by CB1 agonists could involve the stimulation of POMC signaling (Koch et al., 2015). Additionally, our previous study on the effect of RVD-hp(α) on lean rat fed a STD diet displayed a reduction of POMC gene expression (Ferrante et al., 2017). Actually, this could be related to the capability of POMC gene to encode for two peptides, α -MSH and β -endorphin, exerting opposite effects on feeding. On the other hand, CART and NPY gene expressions seem not be involved in the effects on feeding induced by CAF diet or RVD-hp(α) treatment.

In addition, as regards the receptor mechanism at the basis of the observed effects, we also explored the possibility that RVD-hp(α) could modulate transient receptor potential vanilloid 1 (TRPV1) activity, as suggested by Fogaça (2015). The negative results obtained *in vitro* in our laboratory (*data reported in the file:Supplementary data- in vitro and in vivo studies*) indicated that RVD-hp(α) does not directly activate TRPV1

signaling. On the other hand, the blunting effect on hemopressin pharmacological activity induced by co-treatment with TRPV1 antagonists (Fogaça et al., 2015) supports a stimulation of this signaling induced by the peptide. At confirmation of this hypothesis, we performed subsequent *in vitro* studies on HEK293 (human embryonic kidney) cells stably over-expressing recombinant human TRPV1 treated with RVD-hp(α). The tested peptide, despite increasing Ca^{2+} influx in HEK293 cell line, does not seem to be directly involved in TRPV1 activation. On the other hand, a Ca^{2+} influx, although not TRPV1-dependent, could however induce the synthesis on demand of anandamide (Di Marzo et al., 2001; Smart et al., 2000; Zygmunt et al., 2000). To this regard, we performed a further analysis of the pattern of gene expression altered by peptide injection, finding a significant inhibition of fatty acid amide hydrolase (FAAH) gene expression. FAAH is a key enzyme involved in the degradation of endocannabinoids such as anandamide. Anandamide, besides being the principle endocannabinoid, is also the principle endovanilloid molecule. So, our findings of inhibition of FAAH gene expression following peptide treatment, further support the involvement of anandamide in mediating RVD-hp(α) effects *in vivo*.

In conclusion, RVD-hp(α) treatment plays an anorexigenic role paralleled by increased locomotor activity both in STD and CAF diet fed rats. The inhibition of food intake could be partially mediated by lowering of POMC and AgRP gene expression levels in the hypothalamus.

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Declaration of interest

None.

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Legends to figures and table

Fig. 1. Effects of CAF diet and chronic administration RVD-hp(α) (10 nmol) on daily food intake (kcal), recorded every 4 days, in CAF diet fed rats. Rats (n=48) were randomized to standard (STD) diet (n=24) or cafeteria (CAF) diet (n=24) for 14 days, then 14 days treatment with RVD-hp(α) (10 nmol) (n = 12 for each experimental group). Values represent the means \pm S.E.M. (** p < 0.005 vs. STD-vehicle; * p < 0.05 vs. CAF-vehicle; # p < 0.001 vs. STD-vehicle).

Fig. 2. Effects of CAF diet and chronic administration RVD-hp(α) (10 nmol) on the mean daily food intake (kcal) in CAF diet fed rats (n = 12 for each experimental group). Values represent the means \pm S.E.M. (** p < 0.005; *** p < 0.001 vs. STD-vehicle; * p < 0.05 vs. CAF-vehicle).

Fig. 3: Effects of CAF diet and chronic administration RVD-hp(α) (10 nmol) on daily food intake (kcal). The kilocalories were separately calculated for both standard chow and mixture of chips of parmigiano-reggiano cheese, potato chips, roasted, hazelnuts, cookies, curls of salt butter and bits of torrone chocolate. Values represent the means \pm S.E.M. (* p < 0.05 vs. CAF-vehicle).

Fig. 4. Effects of CAF diet and chronic administration RVD-hp(α) (10 nmol) on daily body weight (g), recorded every 4 days, in CAF diet fed rats. Rats (n=48) were randomized to standard (STD) diet (n=24) or cafeteria (CAF) diet (n=24) for 14 days, then 14 days treatment with RVD-hp(α) (10 nmol) (n = 12 for each experimental

group). Values represent the means \pm S.E.M. [* $p < 0.05$; *** $p < 0.001$ vs. STD-vehicle and STD- RVD-hp(α)].

Fig. 5. Effects of CAF diet and chronic administration RVD-hp(α) (10 nmol) on the body weight gain (%) in CAF diet fed rats ($n = 12$ for each experimental group). Values represent the means \pm S.E.M. [*** $p < 0.001$ vs. STD-vehicle and STD- RVD-hp(α)].

Fig. 6. RVD-hp(α) (10 nmol) significantly increased locomotor activity in STD and CAF diet fed rats ($n = 12$ for each experimental group). Horizontal activity and vertical activity were recorded for 10 min. Values represent the means \pm S.E.M. (** $p < 0.005$ vs. STD- and CAF-vehicle).

Fig. 7. Relative gene expression of hypothalamic neuropeptides after RVD-hp (α) (10 nmol) administration in STD and CAF diet fed rats ($n = 12$ for each experimental group), as determined by real-time RT-PCR. Data were calculated using the $2^{-\Delta\Delta C_t}$ method, normalized to β -actin mRNA levels, and expressed as relative to control (calibrator sample, defined as 1.00). Values represent the means \pm S.E.M. (** $p < 0.005$ vs. CAF-vehicle).

Fig. 8. Effects of RVD-hp (α) (1-100 μ M) on TRPV1 channel activity in HEK293 cell line.

Fig. 9: Relative gene expression of hypothalamic FAAH after RVD-hp (α) (10 nmol) administration in STD diet fed rats ($n = 12$ for each experimental group), as determined

by real-time RT-PCR. Data were calculated using the $2^{-\Delta\Delta C_t}$ method, normalized to β -actin mRNA levels, and expressed as relative to control (calibrator sample, defined as 1.00). Values represent the means \pm S.E.M. (** $p < 0.01$ vs. vehicle treated group).

Tab. 1. The numbers indicate the percentage in fat, carbohydrates and protein of STD and CAF diet.

Tab. 2: Effects of CAF diet and chronic administration RVD-hp(α) (10 nmol) on daily food intake (kcal), recorded every 4 days, in CAF diet fed rats. Rats (n=48) were randomized to standard (STD) diet (n=24) or cafeteria (CAF) diet (n=24) for 14 days, then 14 days treatment with RVD-hp(α) (10 nmol) (n = 12 for each experimental group). Values represent the means \pm S.E.M. (# $p < 0.005$ vs. STD-vehicle; * $p < 0.05$ vs. CAF-vehicle; # $p < 0.001$ vs. STD-vehicle).

Tab.3: Effects of CAF diet and chronic administration RVD-hp(α) (10 nmol) on the mean daily food intake (kcal) in CAF diet fed rats (n = 12 for each experimental group). Values represent the means \pm S.E.M. (** $p < 0.005$; *** $p < 0.001$ vs. STD-vehicle; * $p < 0.05$ vs. CAF-vehicle).

Tab. 4: Effects of CAF diet and chronic administration RVD-hp(α) (10 nmol) on daily body weight (g), recorded every 4 days, in CAF diet fed rats. Rats (n=48) were randomized to standard (STD) diet (n=24) or cafeteria (CAF) diet (n=24) for 14 days, then 14 days treatment with RVD-hp(α) (10 nmol) (n = 12 for each experimental

group). Values represent the means \pm S.E.M. [* $p < 0.05$; *** $p < 0.001$ vs. STD-vehicle and STD- RVD-hp(α)].

Tab. 5: Effects of CAF diet and chronic administration RVD-hp(α) (10 nmol) on the body weight gain (%) in CAF diet fed rats ($n = 12$ for each experimental group). Values represent the means \pm SEM. [*** $p < 0.001$ vs. STD-vehicle and STD- RVD-hp(α)].

Tab. 6: RVD-hp(α) (10 nmol) significantly increased locomotor activity in STD and CAF diet fed rats ($n = 12$ for each experimental group). Horizontal activity and vertical activity were recorded for 10 min. Values represent the means \pm S.E.M. (** $p < 0.005$ vs. STD- and CAF-vehicle).

Tab. 7: Relative gene expression of hypothalamic neuropeptides after RVD-hp (α) (10 nmol) administration in STD and CAF diet fed rats ($n = 12$ for each experimental group), as determined by real-time RT-PCR. Data were calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to β -actin mRNA levels, and expressed as relative to control (calibrator sample, defined as 1.00). Values represent the means \pm S.E.M. (** $p < 0.005$ vs. CAF-vehicle).

Table 1. Composition of STD and CAF diet

Component	STD diet 3.20 Kcal/g	CAF diet 4.20 Kcal/g
	%	%
Fat	3	30
Saturated	22.72	45
Insaturated	77.27	55
Carbohydrates	63	56
Protein	14	14
Other components without caloric value	19.5	-

Table 2. Food intake (kcal)

Day	0	4	8	12	16	20	24	28
	Means ±S.E.M.	Means ±S.E.M.	Means ±S.E.M.	Means ±S.E.M.	Means ±S.E.M.	Means ±S.E.M.	Means ±S.E.M.	Means ±S.E.M.
STD-vehicle	78.72±1.0	75.52±1.0	77.76±2.1	75.84±1.2	78.72±1.4	78.08±1.3	75.52±1.0	81.6±1.1
STD-RVD- hp(α)	77.44±1.2	75.52±0.9	78.72±1.0	61.12±1.0 ^{**}	58.88±1.2 ^{**}	63.04±1.1 ^{**}	60.8±0.6 ^{**}	57.6±1.0 [*]
CAF-vehicle	100.8±0.4 [#]	100.8±1.0 [#]	102.9±1.2 [#]	103.32±1.1 [#]	99,12±1.2 [#]	103,32±1.1 [#]	107,52±1.2 [#]	105±1.1 [#]
CAF-RVD- hp(α)	99,96±0.1 [#]	99,96±1.2 [#]	103,32±0.4 [#]	99,12±1.2 [#]	94,92±1.3 [#]	94,08±1.0 ^{#*}	90,72±1.1 ^{#*}	92,4±1.4 [#]

Table 3. Food intake (kcal)

	STD diet	CAF diet
	Means ±S.E.M	Means ±S.E.M
Vehicle	78.72±2.8	103.32±1.8
RVD-hp(α)	57.6±3.8 ^{**}	96.18±1.9 [*]

Table 4. Body weight (g)

Day	0	4	8	12	16	20	24	28
	Means ±S.E.M.	Means ±S.E.M.	Means ±S.E.M.	Means ±S.E.M.	Means ±S.E.M.	Means ±S.E.M.	Means ±S.E.M.	Means ±S.E.M.
STD-vehicle	189.2±2.0	210.5±2.0	217.0±1.1	226±1.2	235.8±1.0	246.4±1.1	258.6±1.0	271.8±1.1
STD-RVD-hp(α)	200.1±1.2	218.1±0.8	225.5±1.0	235.1±1.0	245.0 ±2.0	255.7±1.3	268.4±0.6	282.0±1.0
CAF-vehicle	200.8±0.4	230.8±1.3	249.9±1.2	272.7±1.6	299.5±1.0 [*]	330.4±1.2 ^{**}	365.3±1.0 ^{**}	408.1±1.1 ^{**}
CAF-RVD-hp(α)	220.3±0.7	236.4±1.0	258.9±1.4	282.6±1.2	310.3±1.3 [*]	342.3±1.0 ^{**}	378.2±1.2 ^{**}	424.1±1.0 ^{**}

Table 5. Body weight gain (%)

	STD diet	CAF diet
	Means ±S.E.M	Means ±S.E.M
Vehicle	16.86±3.8	33.1±2.8 ^{***}
RVD-hp(α)	16.84±3.9	34.1±2.7 ^{***}

Table 6. Locomotor activity

	Horizontal Activity	Vertical Activity
	Means \pm S.E.M	Means \pm S.E.M
STD-vehicle	1230.0 \pm 46.8	31.0 \pm 3.8
STD-RVD-hp(α)	1380.0 \pm 20.8 ^{**}	35.2 \pm 2.0 ^{**}
CAF-vehicle	1130.3 \pm 50.8	28.2 \pm 2.9
CAF- RVD-hp(α)	1350.1 \pm 34.0 ^{**}	33.1 \pm 3.9 ^{**}

Table 7. Relative gene expression

	STD-vehicle	STD-RVD-hp(α)	CAF-vehicle	CAF- RVD-hp(α)
	Means \pm S.E.M.	Means \pm S.E.M.	Means \pm S.E.M.	Means \pm S.E.M.
CART	1.00 \pm 0.0	1.10 \pm 0.1	1.09 \pm 0.5	1.08 \pm 0.1
POMC	1.00 \pm 0.0	0.38 \pm 0.0 ^{**}	0.98 \pm 0.1	0.56 \pm 0.2
AGRP	1.00 \pm 0.0	1.15 \pm 0.1	1.80 \pm 0.6 ^{**}	0.98 \pm 0.3
NPY	1.00 \pm 0.0	1.00 \pm 0.2 ^{**}	1.05 \pm 0.2 ^{**}	0.90 \pm 0.1

- In both STD and CAF diet rats, RVD-hp(α) treatment inhibited food intake.
- RVD-hp(α) treatment did not modify body weight in both CAF and STD groups.
- RVD-hp(α) treatment increased locomotor activity in both STD and CAF diet rats.
- RVD-hp(α) treatment decreased POMC gene expression in both STD and CAF rats.
- RVD-hp(α) treatment lowered the elevated AgRP levels induced by CAF diet.